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Assessment of *Herpesvirus saimiri* as a Potential Human Gene Therapy Vector

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Herpesvirus saimiri has characteristics that make it amenable to development as a gene therapy vector. The viral genome is thought to be capable of accommodating large quantities of heterologous DNA while the virus itself can infect many different cell types. Virus infection has been shown in many cases to be persistent by virtue of episomal maintenance in the target cell. In this article we examine the ability of nonselectable recombinant viruses expressing the β-galactosidase gene product to infect a variety of human cells and demonstrate that this virus could be developed as an alternative hematopoietic stem cell gene therapy vector. In contrast to earlier observations, we demonstrate by a number of methods that the virus has the ability to replicate in many human cell types, suggesting the need for the development of a disabled virus for use as a gene therapy vector. ***J. Med. Virol. 57:269–277, 1999.*** © 1999 Wiley-Liss, Inc.

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INTRODUCTION

Large DNA viruses such as *Herpesvirus saimiri* are potentially useful as gene therapy vectors because they are capable of accommodating substantial amounts of additional DNA in their genomes [Smith and Moss, 1983; Ward and Roizman, 1994; Glorioso et al., 1995]. In addition, the persistence of viral DNA may be useful for the gene therapy of stem cells and certain types of differentiated cells with proliferative capacity. This report concerns a reevaluation of *Herpesvirus saimiri* (HVS) in order to better understand its potential as a gene therapy vector.

HVS is the prototype member of the γ2 herpesvirus genus (Rhadinoviruses) [Fleckenstein and Desrosiers, 1982; Jung and Desrosiers, 1994] and shows significant homology at a genomic level with the only known human member of the genus, human herpesvirus 8 or the Kaposi's sarcoma-associated herpesvirus [Chang et al., 1994; Moore et al., 1996]. HVS is apparently apathogenic in its natural host the squirrel monkey (*Saimiri sciureus*) and can easily be isolated from the blood of most individuals [Melendez et al., 1968; Falk et al., 1972]. However, in other nonhuman primates, certain strains are highly oncogenic, producing fulminant T-cell proliferative diseases [Melendez et al., 1972; Fleckenstein, 1979].

HVS strains have been subdivided into three groups (A, B, and C) on the basis of their oncogenic potential and the genetic sequence of the open reading frame encoding the *Herpesvirus saimiri* transformation-associated protein (STP) [Desrosiers and Falk, 1982; Medveczky et al., 1984]. Infection of human blood lymphocytes and thymocytes with strains of subgroup C, in contrast to the other subgroups, yields continuously proliferating T-cell lines with the phenotype of mature CD4- or CD8-positive cells [Biesinger et al., 1992; Mittrucker et al., 1992].

It has been proposed in the past that the virus might be useful as a vector for human cells, and that it appeared to be incapable of growth in a wide range of human cell types [Grassmann and Fleckenstein, 1989; Simmer et al., 1991; Munroe Duboise et al., 1996]. However, most of these experiments made use of virus-infected cells that were positively selected for in culture by virtue of a neomycin resistance gene that was cloned into the virus genome by homologous recombination. In this article we investigate the ability of group A viruses containing the nonselectable marker β-galactosidase to infect a variety of human cells, and reappraise the basic interactions between HVS and hu-

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man cells as a starting point in the development of the virus as a human gene therapy vector.

MATERIALS AND METHODS

Construction of Recombinant Viruses

Plasmid pJCG 111, used to generate HVS A111, was constructed in the following manner. The β-galactosidase gene was inserted into eukaryotic expression plasmid pSA90 (a kind gift from J. Griffiths) downstream of a CMVIE promoter. The 3′ 1.8 kbp of the Kpn E fragment of the HVS genome [Knust et al., 1983] was inserted as an *Nar*I fragment immediately upstream of the CMVIE promoter, resulting in a recombination vector that should insert near the junction of the L and H DNA fragments (i.e., just within the repeat sequences). This strategy of a single crossover recombination event is essentially as described by Grassmann and Fleckenstein [1989]. The recombination vector used to generate HVSΔORF4 was constructed by adding *Bgl*II linkers to a *Pst*I fragment containing the CMVIE β-galactosidase cassette and ligating directly into a *Bgl*II site (position 11769) within ORF4, which had previously been cloned as a component of the *Kpn*I B fragment of the genome [Knust et al., 1983]. This vector was designed to create a double crossover event. The recombination vector used to generate HVSΔORF16, also through a double crossover event, was constructed by PCR amplifying ORF 16 and flanking sequences from ORF15 (forward primer sequence 5'→3' GCCGAATCCCACAGTGCCAAGCTTGCCAGTT, reverse primer sequence 5'→3' CGCCTGCAGGGTGTATAACTGAGTGTACAGC) and ORF17 (forward primer sequence 5'→3' GGGCTGCAGGCTGTACACTCAGTTATACACC, reverse primer sequence 5'→3' CCCGCATGCACTTGATCCAGGACATGCTTC) using PCR primers that introduced a *Pst*I restriction site within ORF16. The PCR conditions used were 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. These fragments were then cloned into pUC18 and sequenced to verify their integrity (data not shown). The *Pst*I fragment containing the CMVIE β-galactosidase cassette was then ligated directly into this site.

Recombinant virus was generated by performing a cotransfection with 1 μg of infectious wt virus DNA (HVS A11) and 10 μg of the appropriate plasmid. This was carried out in low passage Owl Monday Kidney (OMK) cells using the calcium phosphate method and a glycerol shock. The cells were incubated for 7 days to allow the development of extensive cytopathic effect (cpe), after which time the supernatant was harvested and used to infect new monolayers of OMK cells. β-galactosidase expressing viruses were selected for using X-Gal staining and an agar overlay. Blue plaques were picked and purified in this manner until a genetically pure stock of the virus was obtained. Proof that the gene had been correctly inserted was demonstrated by PCR from a site in the virus genome outside of the homologous recombination sequence to a site within the expression cassette.

Cells

Primary cell cultures of bone marrow (from normal donors) and mobilized peripheral blood (from adult cancer patients who had received cyclophosphamide at 2–4 g/m^2 and GSF at 4–8 μg/kg) were enriched for CD34$^+$ cells by using the MACS magnetic cell sorting CD34 progenitor cell isolation kit (Militenyi Biotec, Bergisch Gladbach, Germany) and subsequently analyzed by flow cytometry to determine the percentage of progenitor cells. Cultures of progenitor cells were maintained in methylcellulose medium for 2 weeks, after which colonies were counted and analyzed.

The human cell lines used in experiments were 293T (transformed primary embryonal kidney), A549 (lung carcinoma), K562 (chronic myelogenous leukemia), HT-29 (colonic adenocarcinoma), Jurkat (T-cell lymphoma), Molt-4 (T-cell leukemia), and Raji (Burkitt's Lymphoma).

Infection of Cells With HVS

In order to reduce variability, all cell samples that were infected in the quantitative β-galactosidase assay were infected in suspension (whether they were monolayer or suspension cells). Typically, this involved the incubation of 5×10^5 cells with approximately 1×10^5 virus particles (i.e., moi of 0.2) in around 0.5 ml of medium for 2 hr at 37°C. The volume was then made up to 2.5 ml with fresh medium and the cells added to a 35-mm tissue culture dish. Incubation was continued for a further 48 hr before subsequent analysis. In other experiments, infection of monolayers was carried out using conventional methods.

Primary cultures enriched for CD34$^+$ cells were infected by centrifuging about 5×10^4 cells with 1–5×10^5 virus particles in a minimal volume for 2 hr at 2,000 rpm. This was found to produce a greater number of infected cells than merely incubating the cells and virus together, as described above. Similar techniques are often used for retroviral infection of stem cells [Bahnson et al., 1995].

Immunofluorescence

Immunofluorescence was carried out on infected cells using the following method. Cells to be infected were grown on glass coverslips in six-well tissue culture dishes. The cells were infected with approximately 0.1 moi of HVS A11 and were incubated at 37°C until cpe developed (usually after 3–4 days). The cells were fixed with 50% (v/v) methanol 50% (v/v) acetone for 30 sec allowing total cell staining. The coverslips were washed three times in PBS before being blocked in 1% (w/v) full fat milk powder for 1 hr at 37°C. The coverslips were then washed three times in PBS and 100 μl of diluted primary antibody were placed directly on top of the cells on the coverslip, which was then incubated at 37°C for 1 hr. MAb SB [Randall et al., 1984] was used at a 1/200 dilution, while ORF 51 preimmune and immune rabbit antisera were used at a 1/250 dilution. Commercially available anti–β-Galactosidase Mab was

used at a 1/1,000 dilution. The coverslip was again washed three times in PBS and 100 μl of a 1/200 dilution in PBS of a fluorescein (FITC)-conjugated antibody (Sigma, St. Louis, MO) were added for 1 hr at 37°C. The coverslip was again washed three times in PBS and then once in distilled water before being mounted, inverted, on a microscope slide using a drop of Vector Shield mounting medium (Vector Laboratories, Burlingame, CA). The slides were examined using a Zeiss Axiovert 135TV inverted microscope with a Neofluar 40× oil immersion lens. Fluorescence images were obtained using a 35-mm camera adapter loaded with 100 ASA Ektachrome film (Kodak).

Recovery Assay

Cell cultures were grown up in six-welled dishes and infected with virus at a moi of 0.1. Cultures were left for 5 days at 37°C, after which 1 ml of supernatant was removed from each well and centrifuged for 5 min at 3,000 rpm in a bench top centrifuge to remove any cellular debris. The sample was then added to a monolayer of OMK cells in a six-welled dish, which was left until the development of cytopathic effect/plaques was observed.

Infectious Center Assay

1×10^6 cells (293T, K562, Raji) were infected at a moi of 1 with wild-type HVS A11 by mixing in a small volume of medium (0.5 ml) and spinning for 90 min at room temperature (2,000 rpm). The cell/virus suspension was then serially diluted and mixed with OMK cells before being plated out in a six-welled dish. Carboxymethylcellulose was included in the medium to prevent infection of the monolayer by cell-free virus and secondary plaque formation. After 1 week the monolayers were examined (along with appropriate controls) for the presence of plaques. This allowed the number of infected human cells that were capable of producing infectious virus (thereby producing a plaque in the monolayer) to be determined.

β-Galactosidase Activity Assay

Three days after infection with HVS A111 cells were washed in PBS and lysed by the addition of lysis buffer (25-mM Tris-phosphate pH 7.75, 8-mM $MgCl_2$, 15% glycerol, 0.1-mM EDTA, 0.1% Triton-X100, 1-mM DTT); 200 μg of protein, as measured by the dotMetric protein assay (Geno Technology, St. Louis), was added to an Eppendorf tube and the volume made up to 300 μl with Z-buffer (60-mM Na_2HPO_4, 40-mM NaH_2PO_4, 10-mM KCl, 0.36% β-mercaptoethanol, 1-mM $MgSO_4$); 60 μl of O-Nitrophenyl β-O- Galactopyranotide (ONPG) solution was added (60-mM Na_2HPO_4, 40-mM NaH_2PO_4, 4% ONPG). The sample was then incubated for 2 hr at 37°C, after which the reaction was stopped by the addition of 250 μl of Na_2CO_3. The OD_{420nm} was then read against appropriate controls.

X-Gal Staining of Cells

OMK cells were normally stained using an agar/Eagle's medium overlay containing 0.01% X-gal and incubating at 37°C for 1–2 days. Primary hematopoietic cell cultures were stained by carefully adding Eagle's medium containing 0.02% X-gal onto the methylcellulose after colony development and incubating at 37°C for 2–3 days.

Detection of Linear Virus DNA in Infected Cells

Pulsed field gel electrophoresis (PFGE) was carried out using a Biorad Chef DR-III system and eletrophoresis cell. The run conditions used were designed to separate species of between 50 and 500 kbp: buffer 1 × TAE, run time 19 hr 24 min, initial switch time 5.8 sec, final switch time 38.5 sec, voltage gradient 6 V/cm, angle 120°, temperature 14°C, 1% pulsed field certified agarose. Agarose plugs were prepared by pelleting approximately 2×10^6 cells and washing twice in PBS. The cells were then resuspended in 200 μl of low-melting-point agarose and cast into wells of a 100 μl plug former. After they had set, the plugs were incubated overnight in 1 ml of 1% laurylsarcosine in 0.5-M EDTA (pH 8.0) with proteinase K at a concentration of 100 μg/ml at 37°C. Next day the plugs were washed 5 times in TE (pH 8.0) at 37°C for 10 min each wash and stored at 4°C until use in PFGE.

The gel was Southern-blotted onto nitrocellulose using standard methods and probed using the *Eco*D fragment of the HVS genome. The probe was labeled with α^{32}P dCTP using the random priming method (Megaprime kit, Amersham, Arlington Heights, IL).

RESULTS

(1) Recombinant HVS is capable of transferring the β-galactosidase marker gene to a wide variety of human cell lines, but expression of the marker gene is not maintained on continued passage of the virus.

β-galactosidase expressing HVS A111 was engineered by homologous recombination of the marker gene to a site within the repeat region at the end of the genome. The procedure utilized a single crossover event essentially as described for the neomycin resistance gene by Grassman and Fleckenstein [1989]. During the isolation of this virus by plaque purification, we noticed that the amount of β-galactosidase expression (as evidenced by X-gal staining) varied within stocks of virus that were genetically pure by Southern blotting (data not shown), and that continued passage of the virus resulted in an increasing ratio of white to blue plaques. This phenomenon was also noted with HVS insertion mutants in ORF4 and ORF16 produced by the insertion of a β-galactosidase expression cassette. Figure 1 outlines the method used to construct the viruses, and example of a PCR reaction performed to demonstrate the correct positional cloning of the β-galactosidase cassette (for HVS A111). Figure 2A demonstrates transfer of β-galactosidase to OMK cells by HVSΔORF4 by immunofluorescence; Figure 2B shows

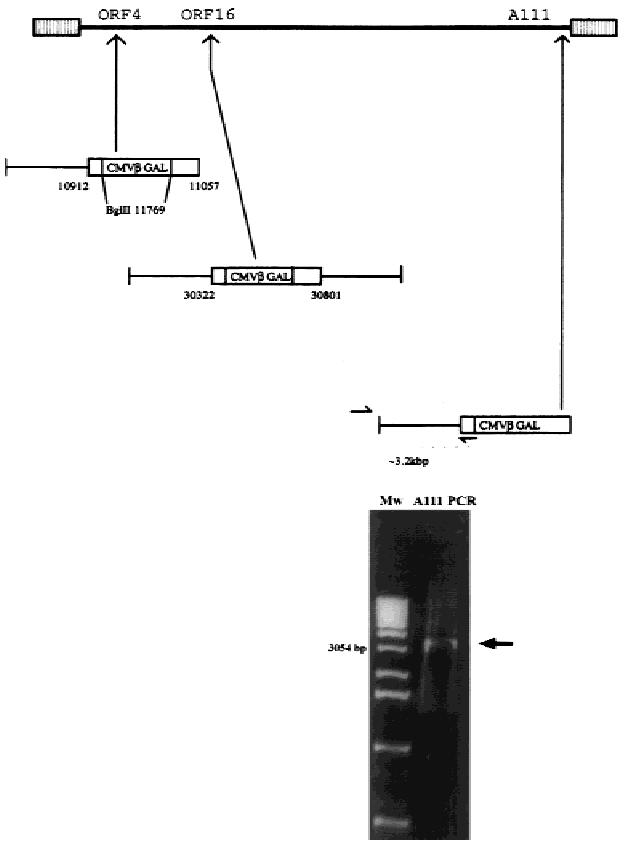

Fig. 1. Construction of recombinant viruses. The unique region of the HVS genome is represented by a solid line and the terminal repeat sequences by boxes containing vertical lines. The position of insertion of the CMVIE β-galactosidase cassette is indicated for each of the three recombinant viruses. The methods used to construct each of the recombinant viruses are described above. Proof that the gene had been correctly inserted was demonstrated by performing PCR from a site in the virus genome outside the homomlogous recombination sequence to a site within the CMVIE β-galactosidase cassette. The PCR product generated from HVS A111 DNA is included in the figure as an example, along with the position of the primers relative to the insert (forward primer sequence 5'→3' CAGCTACTGATACTGGGTGT-GAAGGGCATG, reverse primer sequence 5'→3' GTTGCGCAGCCT-GAATGGCGAATGGCG. PCR conditions, 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 3 min, and a final extension at 72°C for 10 min).

the same plaque stained for HVS structural protein encoded by ORF51; whereas Figure 2C and D shows examples of blue and partially blue plaques produced in the same well of OMK cells by this virus following X-gal staining (the well also contained clear plaques).

Cells of a number of different lineages were tested for β-galactosidase expression following infection with early passage stocks of HVS A111 (Fig. 3). In this quantitative assay, absorbance readings were taken from both mock-infected and HVS A111-infected cells. The average of two separate mock-infected readings was subtracted from the average of two separate virus-infected readings to calculate the amount of β-galactosidase activity due to the recombinant virus. Infections were carried out simultaneously with all cell lines using the same stock of virus. Control experiments demonstrated that wild-type virus had no effect on the levels of endogenous β-galactosidase activity present in the cells (data not shown). In general, cells that had the greatest potential to support the replication of HVS produced the highest levels of β-galactosidase activity. The absorbance readings obtained for the various cell lines are illustrated in Figure 3. 293T cells gave by far the highest reading of the human cells, but all of the cell lines tested showed the capacity to be infected by the virus and express the β-galactosidase gene that it carried. As a control, OMK cells (in which the virus is routinely grown) were infected with HVS A111 and showed a fivefold increase in the levels of β-galactosidase activity compared with 293T cells (data not shown).

(2) HVS is capable of transferring the β-galactosidase gene to hematopoietic progenitor cells, and the gene product is apparently expressed in progeny cells.

Upon staining with X-gal, cultures derived from bone marrow and mobilized peripheral blood and infected with early passage HVSΔORF4 contained a few blue colonies. Some of these comprised of only a few cells, suggesting that the differentiation process had been halted by cytopathic effect produced by virus or expressed β-galactosidase. A few colonies, however, appeared to have developed normally and expressed β-galactosidase in all of the cells. All of these colonies resembled CFU-GM colonies morphologically. Figure 4A shows a blue CFU-GM colony interspersed between two normal colonies of the same type, while Figure 4B shows a close up of the same colony.

(3) Evidence for replication of HVS in a wide variety of human cells are as follows.

Immnofluorescence IF analysis was performed on HVS-infected cells using two different antibodies. Monoclonal antibody SB recognizes the immediate early virus gene product of ORF 57, while polyclonal antibody 51 recognizes the envelope protein encoded by ORF 51 (likely to be a late gene product). Raji, Jurkat, Molt4, K562, 293T, and A549 cells were all analyzed and showed evidence of expression of both ORF 57 and ORF 51 gene products. Figure 5A shows an example of a monolayer (A549) cell line stained with antibody SB, and Figure 5B shows the same cell line stained with polyclone 51. In the case of monolayer cell lines, groups of cells expressing the proteins were often seen clustered together, suggesting the cell-to-cell spread of the gene products in a manner resembling plaque formation.

Southern Blotting Detection of viral genomes was accomplished by the probing Southern blots of pulsed field agarose gels of HVS-infected/mock-infected cell lines with a radioactively labeled fragment of the HVS genome. The presence of a band comigrating with a linear genomic DNA control would strongly suggest the production of virus particles (i.e., packaged DNA). Figure 6 shows that the *Eco*D probe hybridized to a band that comigrated with linear genomic DNA in all of the infected cell samples, whereas none of the uninfected controls produced a signal of this size. 239T and A549 cells produced the strongest signal for linear ge-

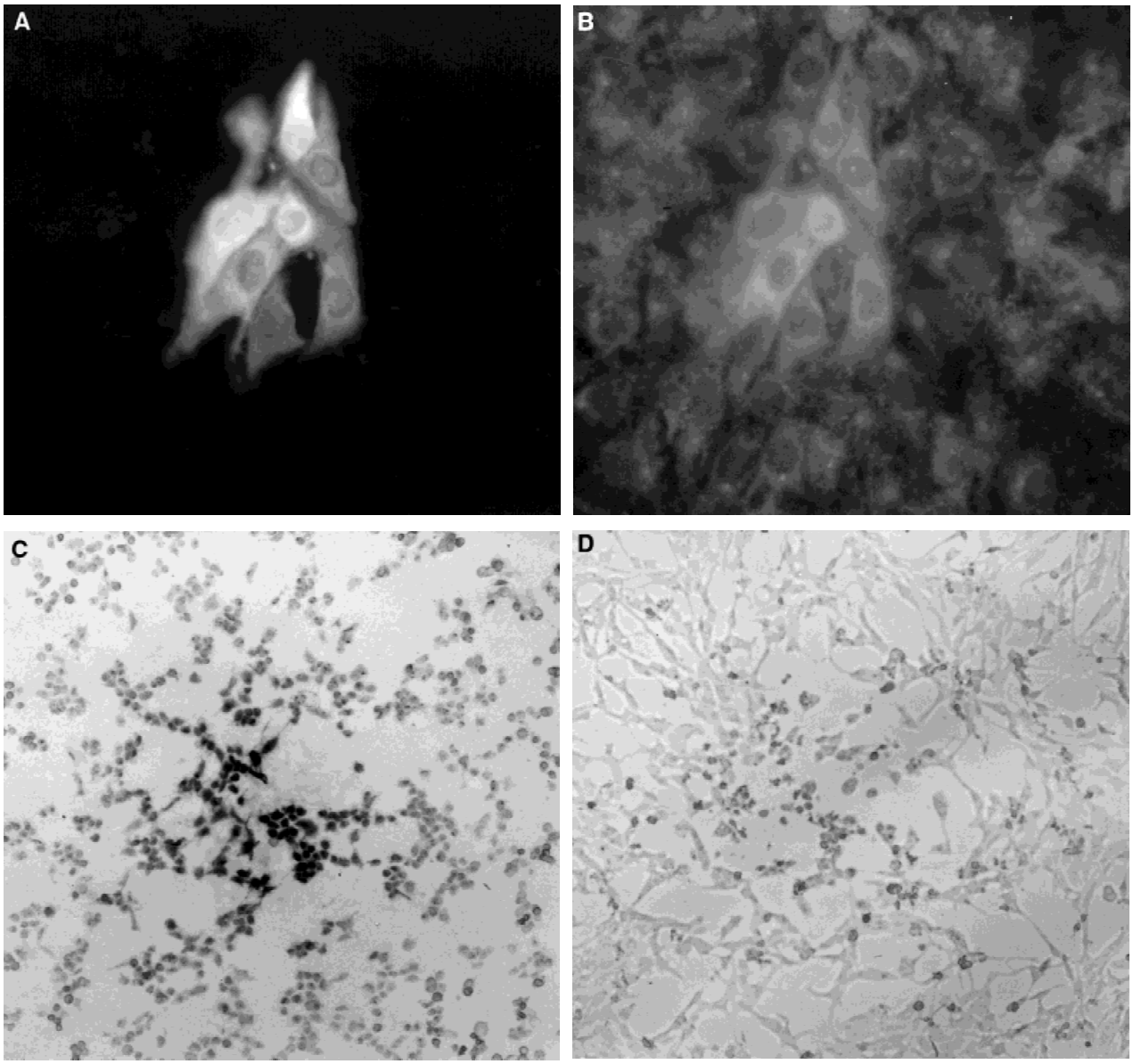

Fig. 2. β-galactosidase expression from recombinant HVS in OMK cells. **A:** Detection of β-gal immunoreactivity using fluorescein-conjugated secondary antibody in a plaque produced by HVSΔORF4 in OMK cells. **B:** Detection of virus structural protein (ORF 51 gene product) in the same plaque using Texas red conjugated secondary antibody. **C:** Blue plaque produced by HVSΔORF4 in OMK cells stained with X-gal. **D:** Partially blue plaque produced in the same well.

nomic DNA, and also produced a strong signal with DNA of an extremely high molecular weight (indicated on the figure), which was also faintly detectable in the lane containing infected K562 cells. 293T and A549 cell lines also gave a very strong signal with the well containing the plug of cells (indicated on the figure as well DNA).

Virus Recovery Assay In order to assess the ability of human cell lines to produce infectious virus, an aliquot (0.5 ml) of medium was removed 5 days after the initial infection and used to infect a fresh monolayer of OMK cells. Development of cytopathic effect indicated the presence of infectious virus particles. As a control, medium containing virus was incubated for an equivalent length of time at 37°C and used to infect OMK cells. No cpe was produced, indicating that the input virus had been inactivated. Supernatants from all cell lines analyzed (i.e., K562, Molt4, Jurkat, Raji, 293T, A549) produced varying amounts of cpe on the OMK monolayers, indicating active virus replication. Figure 7A shows the control and Figure 7B the Molt4 recovery assays as examples.

Infectious Center Assay Infectious center assays were employed to determine quantitatively the capacity of three different human cell lines to produce infectious virus. It was found that 293T cells generated nearly twice as many plaques as K562 cells and roughly three times as many as the Raji cell line (Table

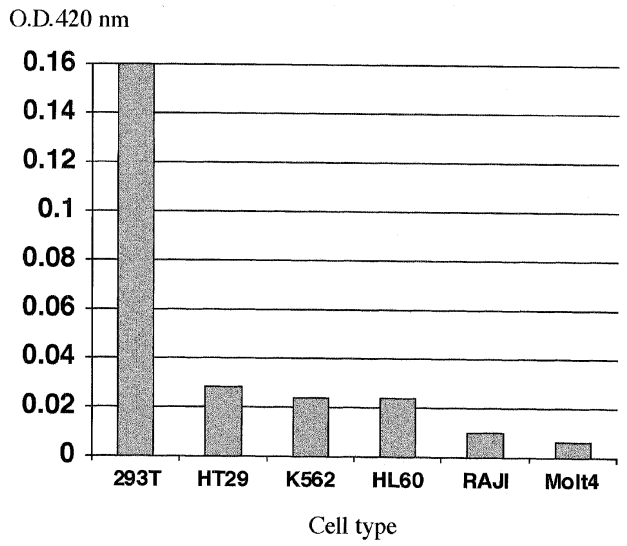

Fig. 3. Qualitative β-galactosidase assay of different human cell lines infected with early passage HVS A111. All procedures were carried out as described in Materials and Methods, with readings being taken 48 hr after infection. The bars indicate the mean value from two separate readings of infected cells minus the equivalent readings from uninfected controls. All cells were infected at the simultaneously with equivalent amounts of virus.

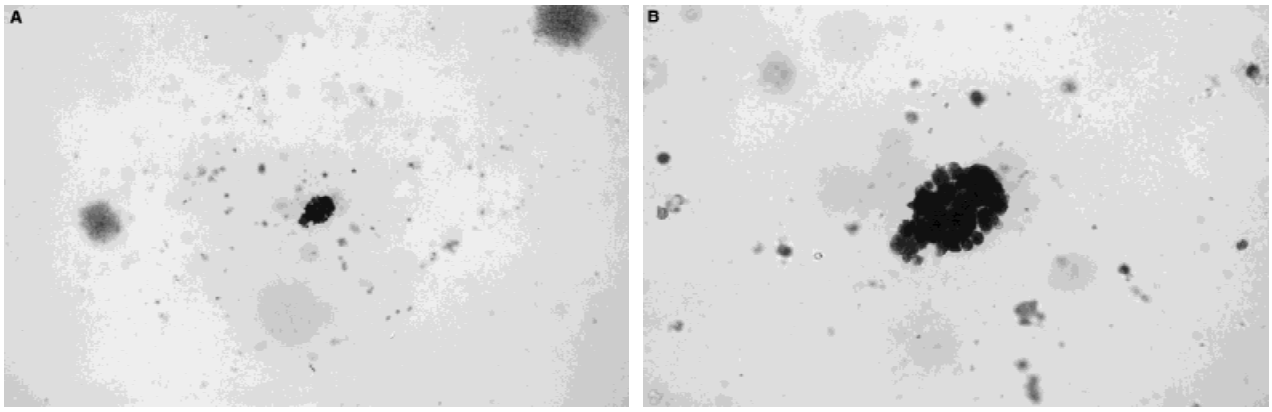

Fig. 4. Transfer of β-galactosidase to a human progenitor cell and evidence of gene expression in differentiated progeny. A primary mobilized peripheral blood culture was enriched for $CD34^+$ cells and infected with HVSΔORF4 as described in Materials and Methods. After 2 weeks in methylcellulose culture, the cells were overlaid with Eagle's medium containing X-gal and incubated until blue cells were evident. **A:** ×100 magnification showing blue CFU-GM colony between apparently uninfected colonies. **B:** ×320 magnification of the same colony showing uniformity of β-galactosidase expression.

I). Controls were employed to ensure that extracellular virus was incapable of infecting the OMK monolayer (because of the presence of CMC) and to examine what effects, if any, uninfected cells had on the OMK cells.

293T cells sometimes induced a characteristic cytopathic effect when in contact with the OMK cells, but this was distinguishable from normal plaque formation.

DISCUSSION

We have investigated the potential development of *Herpesvirus saimiri* as a gene therapy vector. Earlier publications have demonstrated that selectable virus has the ability to persist in a variety of human cell lines for long periods of time [Grassmann and Fleckenstein 1989; Simmer et al., 1991], apparently without the production of infectious progeny (in all but a very few cases). The experiments reported here were conducted to determine the characteristics of the virus that would be relevant in future gene therapy applications. These included determining which human cell types the virus infected most efficiently, demonstrating the transfer of a nonselectable marker gene to cells, and investigating whether wild-type virus has the ability to grow in a variety of human cells (important for the design of a disabled virus). As an initial step, a recombinant virus was generated in which the β-galactosidase gene was inserted between the L and H components of the genome (at a site that is not transcribed and does not overlap with a major open reading frame [Bankier et al., 1985; Stamminger et al., 1987]) and its ability to transfer this gene to a number of different human cell lines was investigated. Viruses in which ORFs 4 and 16 were disrupted by insertion of the CMVIE β-galactosidase cassette were also generated.

An interesting characteristic shared by all of the viruses was the apparently mixed phenotype with regard to blue/white-color selection and the fact that contin-

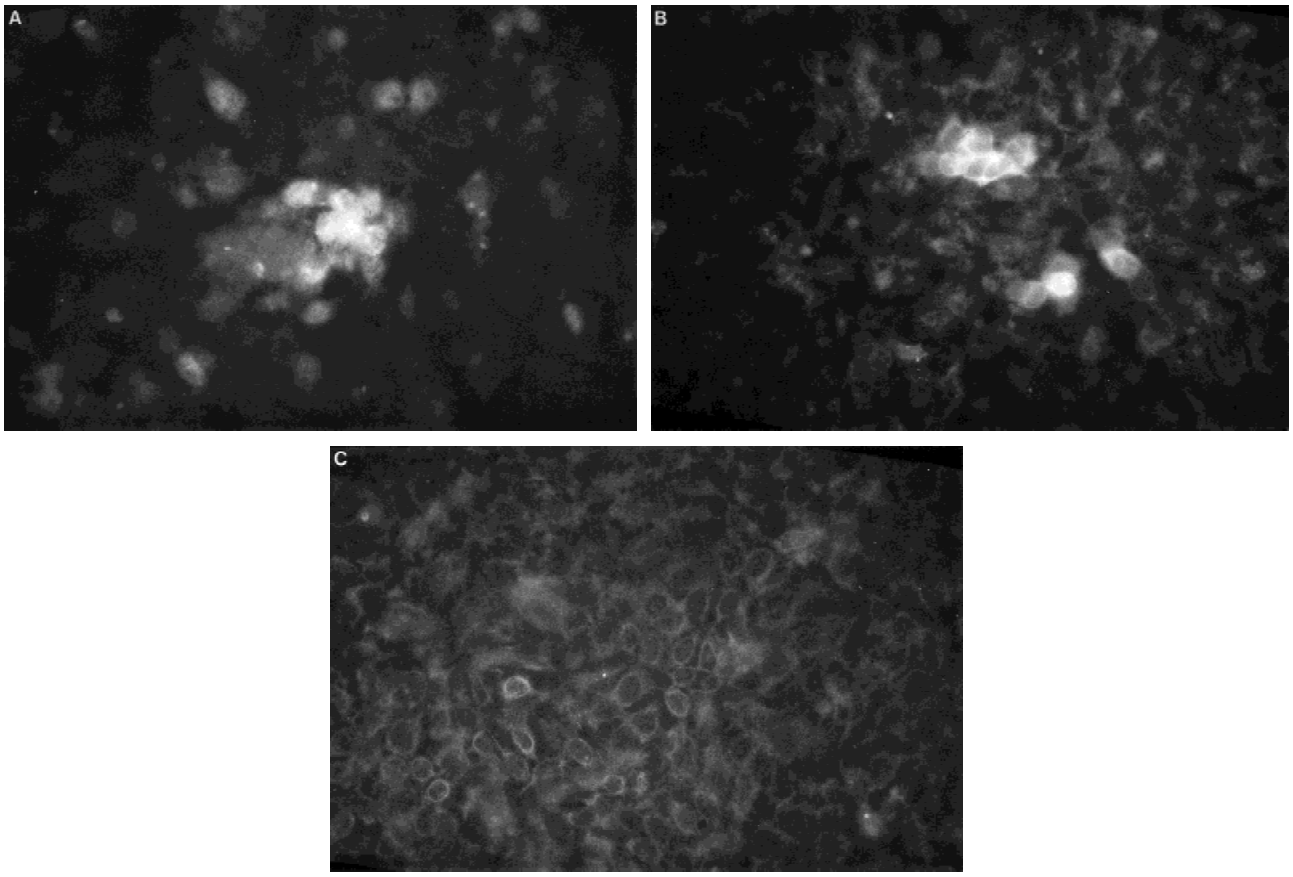

Fig. 5. Detection of HVS gene products in infected A549 cells by immunofluorescence. Cells were infected with HVS at a moi of 0.1 and incubated for 4 days. Standard immunofluorescence was then performed using primary antibodies targeted to (**A**) the immediate early gene product of ORF57 and (**B**) the structural protein encoded by ORF51. Clusters of positively staining cells could be seen in each case. Both antibodies were also used to probe mock-infected cells as a control (**C**).

ued passage resulted in an increase in the proportion of clear plaques (Fig. 2). Repeated genetic analysis (Southern blotting and PCR) of different plaque isolates revealed that the β-galactosidase gene was present in all cases but that somehow expression was downregulated over time. To our knowledge, this phenomenon has not been reported in the limited work carried out on recombinant HVS, but we note that no other group has utilized this marker gene/promoter combination. Further investigations showed that treatment of recombinant virus-infected OMK cells with 5-azacytidine (an inhibitor of DNA methylation) resulted in a small reproducible increase in β-galactosidase activity, suggesting that methylation might be at least partially responsible for the repression of β-galactosidase expression (data not shown). We note that HVS ORF27 has been shown to contain motif that is conserved in functionally characterized methylases [Albrecht et al., 1992]. We also investigated the possibility that the CMVIE promoter might be downregulated by HVS infection using a transfection/superinfection experiment. However, contrary to our hypothesis, we found that expression of β-galactosidase was markedly increased by HVS infection (data not shown).

Quantitative β-galactosidase assays revealed that the virus had the capacity to transfer a functional gene to all cell lines tested but that the amount of enzyme activity varied. The most likely explanation is that the virus was more efficient at infecting some cell lines than others. Indeed, the cell lines that gave the higher levels of activity/unit of protein also exhibited the higher ratios of blue to white cells (data not shown). Although it could be argued that CMV promoter activity might vary between cell lines, thereby influencing the amount of enzyme present in each infected cell, it would probably be unlikely to generate the differences observed. 293T cells produced a far higher reading than the other cell lines; HT29, K562, and HL60 cells all produced approximately equivalent readings, while Raji and Molt4 cells produced the lowest. At first inspection, these results are somewhat surprising in that HVS normally resides in the T-cells of its natural host [Fleckenstein and Desrosiers, 1982] and it would seem reasonable to assume that these cells might be most amenable to infection with the virus. However, our results suggested that under the conditions of the experiment T-cells (Molt4) were the least infectable of the cell lines tested. 293T cells, the cells that produced the highest levels of enzymatic activity, are originally derived from human embryonic kidney cells. The virus is

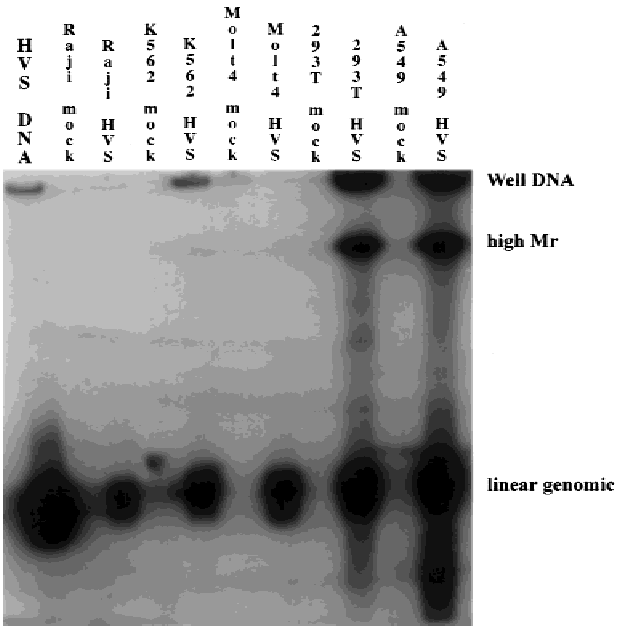

Fig. 6. Detection of viral DNA in HVS A11-infected human cells. Cell plugs were subjected to pulsed field gel electrophoresis and Southern blotting as described in Materials and Methods. The blot was probed with the *Eco*D fragment of the *Herpesvirus saimiri* genome and exposed to X-ray film. A sample of viral DNA was also run on the gel as a marker. The hybridizing species are identified as linear genomic, high Mr, and well DNA.

routinely grown in owl monkey kidney cells in the laboratory and it seemed possible that the 293T cells might be supporting a degree of virus replication (thereby causing cell-to-cell spread of the virus and contributing to the enhanced β-galactosidase levels).

In order to test the hypothesis that human cell lines might be able to support virus replication, we carried out a series of different investigations. Immunofluorescence experiments revealed that all cell lines demonstrated expression of both an immediate early and a late gene product. This information alone dictated that a virus disabled in an essential gene will be constructed for future applications.

Southern blot analysis of pulsed field gels revealed the presence of linear virus genomes (i.e., packaged into virions) in all infected cell lines. This also strongly indicated the production of virus. 293T, A549, and, to a lesser extent, K562 cells also showed the presence of a hybridizing species much further up the gel and another that corresponded to well DNA. Although it could be argued that the well DNA signal might be generated by DNA associated with incompletely digested cells, these signals are likely to represent extremely large DNA species (possibly complex replicating structures). It is interesting to note that these DNA species are associated with the cell types (i.e., k562 and 293T) that produced the highest reading on the β-galactosidase assay, indicating that these readings might be related to the degree of replication occurring. Earlier work [Grassmann and Fleckenstein, 1989; Simmer et al., 1991] used Gardella gels to distinguish between linear

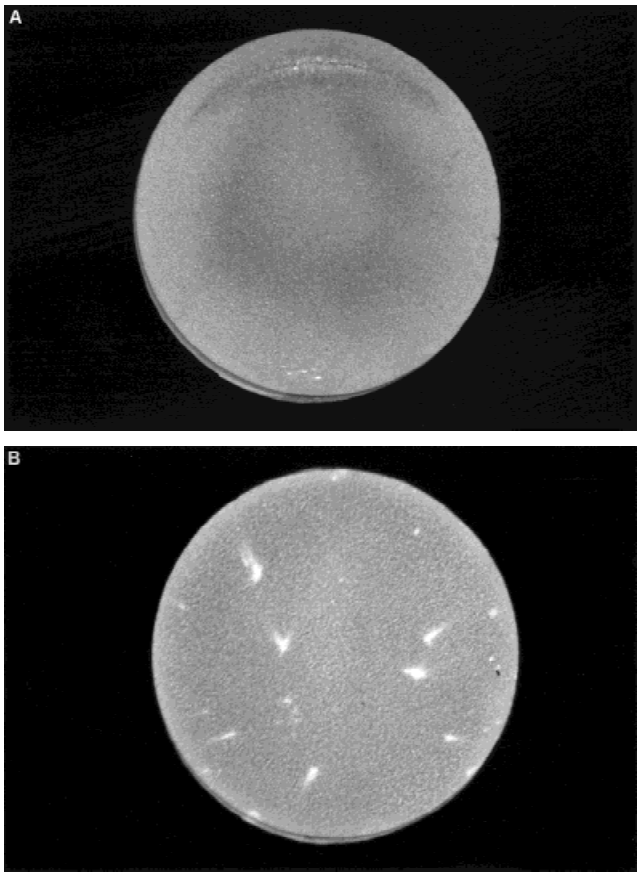

Fig. 7. Virus recovery assay. Supernatant was taken from virus-infected human cell lines and used to infect OMK monolayers to test for the presence of infectious virus particles. After the development of cpe the monolayers were stained with crystal violet and photographed: **A:** control supernatant; **B:** Molt 4 supernatant.

TABLE I. Infectious Center Assay[a]

Cell type	Number of infectious centers produced
Raji	4.8×10^4
K562	8.0×10^4
293T	1.4×10^5

[a]Human cell lines Raji, K562, and 293T were infected with wt HVS and the number of infectious virus particles produced assessed by plaque formation in a cocultivated monolayer of OMK cells. The table gives the number of infectious centers produced per 10^6 infected cells (infected at a multiplicity of 1).

and episomal forms of the virus genomes. The great majority of the cell lines that were persistently infected with the selectable recombinant viruses only showed evidence of the episomal form of DNA. In these experiments, although some of the cell lines that we used were identical to those employed by the other groups, we never detected reproducible signals corresponding to this form of DNA. In order to explain this apparent anomaly we would suggest that while a small proportion of the cells infected with the virus go on to become persistently infected, many of the cells are capable of supporting active replication of the virus. The original work would not have detected this initial production of

virus, selecting as it did for surviving cells that expressed the neomycin resistance gene.

The virus recovery assay provided conclusive proof that infectious virus was being produced by all the cultures tested, while the infectious center assay quantitatively demonstrated that the number of virus producing cells differed between cell lines (although since this assay was unable to determine how many virus particles individual cells produced, an accurate titer for virus production could not be calculated).

Perhaps the most interesting observation made during the course of this study was the ability of HVS to transfer the marker gene β-galactosidase to committed primary hematopoietic progenitor cells. It was impossible to determine the exact percentage of cells that were transduced by the virus because none of the virus stocks generated in this study were capable of producing 100% blue plaques. Also, it is unclear whether a proportion of infected cells were killed by the virus. Nevertheless, this essentially qualitative observation is extremely encouraging for the development of HVS as a gene therapy vector for hematopoietic cells. For stem cell gene therapy, sustained expression of the transgene is a prerequisite for success. Many investigators consider that only those gene delivery vehicles that ensure stable integration into the host DNA, i.e., retroviruses and possibly adeno-associated viral vectors, are applicable. However, with these studies we have shown that HVS may provide an alternative. Long-term assays will be required to determine if the transduction of more primitive progenitors is also possible.

It is concluded that *Herpesvirus saimiri* is potentially useful as a gene therapy vector because of its ability to infect a wide variety of human cells and persist as a replicating episome as demonstrated by others [Grassmann and Fleckenstein, 1989; Simmer et al., 1991]. However, the above investigations show that question marks remain over its ability to maintain heterologous nonselectable gene expression long-term from a commonly used promoter/reporter gene cassette, and that the wild-type virus is capable of replicating in a wide variety of human cells, necessitating the development of a disabled mutant. Future studies will attempt to address these issues.

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